The sodium chloride cotransporter SLC12A3: new roles in sodium, potassium, and blood pressure regulation

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Abstract: SLC12A3 encodes the thiazide-sensitive sodium chloride cotransporter (NCC), which is primarily expressed in the kidney, but also in intestine and bone. In the kidney, NCC is located in the apical plasma membrane of epithelial cells in the distal convoluted tubule. Although NCC reabsorbs only 5 to 10 % of filtered sodium, it is important for the fine-tuning of renal sodium excretion in response to various hormonal and non-hormonal stimuli. Several new roles for NCC in the regulation of sodium, potassium, and blood pressure have been unraveled recently. For example, the recent discoveries that NCC is activated by angiotensin II but inhibited by dietary potassium shed light on how the kidney handles sodium during hypovolemia (high angiotensin II) and hyperkalemia. The additive effect of angiotensin II and aldosterone maximizes sodium reabsorption during hypovolemia, whereas the inhibitory effect of potassium on NCC increases delivery of sodium to the potassium-secreting portion of the nephron. In addition, great steps have been made in unraveling the molecular machinery that controls NCC. This complex network consists of kinases and ubiquitinases, including WNKs, SGK1, SPAK, Nedd4-2, Cullin-3, and Kelch-like 3. The pathophysiological significance of this network is illustrated by the fact that modification of each individual protein in the network changes NCC activity and results in salt-dependent hypotension or hypertension. This review aims to summarize these new insights in an integrated manner while identifying unanswered questions.

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The sodium chloride cotransporter SLC12A3:

new roles in sodium, potassium, and blood pressure regulation

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SLC12A3 encodes the thiazide-sensitive sodium chloride cotransporter (NCC), which is primarily expressed in the kidney, but also in intestine and bone. In the kidney, NCC is located in the apical plasma membrane of epithelial cells in the distal convoluted tubule. Although NCC reabsorbs only 5 to 10% of filtered sodium, it is important for the fine-tuning of renal sodium excretion in response to various hormonal and non-hormonal stimuli. Several new roles for NCC in the regulation of sodium, potassium, and blood pressure have been unraveled recently. For example, the recent discoveries that NCC is activated by angiotensin II but inhibited by dietary potassium sheds light on how the kidney handles sodium during hypovolemia (high angiotensin II) and hyperkalemia. The additive effect of angiotensin II and aldosterone maximizes sodium reabsorption during hypovolemia, whereas the inhibitory effect of potassium on NCC increases delivery of sodium to the potassium-secreting portion of the nephron. In addition, great steps have been made in unraveling the molecular machinery that controls NCC. This complex network consists of kinases and ubiquitinases, including WNKs, SGK1, SPAK, Nedd4-2, Cullin-3 and Kelch-like 3. The pathophysiological significance of this network is illustrated by the fact that modification of each individual protein in the network changes NCC activity and results in salt-dependent hypotension or hypertension. This review aims to summarize these new insights in an integrated manner while identifying unanswered questions.

Keywords: Aldosterone; Angiotensin II; Hypertension; Tacrolimus; Thiazide; WNK kinase.

Typical hallmarks of NCC

The presence of a sodium chloride cotransporter was first suggested in urinary bladder of the winter flounder [95, 96]. Subsequent studies in this tissue demonstrated that this cotransporter could be inhibited by thiazide diuretics [111] and the cDNA encoding this transporter was isolated [31].

Studies using micropuncture and isolated perfused tubules had identified the same pharmacological and kinetic transport characteristics in the early distal convoluted tubule (DCT) of rat kidney [23, 60]. Indeed, Gamba and colleagues succeeded in isolating cDNA of the sodium chloride cotransporter from rat kidney [30]. NCC is encoded by the *SLC12A3* gene (55-kb, 26 exons) and belongs to the SLC12 family of electroneutral cation chloride cotransporters [29]. Besides the kidney, NCC was shown to be expressed in intestine and bone where it is likely involved in sodium and calcium absorption [4, 20]. It has been suggested that NCC is expressed in various other tissues but this has not been confirmed [29]. In the kidney, NCC is located in the early part of the DCT (also called DCT1) (**Figure 1**), **but gradually decreases along the later** part of the DCT (DCT2), where it co-localizes with the epithelial sodium channel (ENaC) [3]. The sodium chloride cotransporter (NCC) mediates the reabsorption of sodium across the apical membrane of the DCT (**Figure 1**) [23]. Chronic activation or inhibition of NCC is usually accompanied by morphological changes in the DCT resulting in hypertrophy or atrophy [21, 61, 66, 126]. The gradient required for sodium chloride transport through NCC is generated and maintained by the basolateral sodium-potassium ATPase pump. The potassium that enters the cell via this pump is **recycled by basolateral potassium transporters. In addition, potassium may also be** secreted apically by the renal outer medullary potassium channel (ROMK) and a potassium chloride cotransporter [133]. In addition, NCC modulates transcellular magnesium and calcium reabsorption in the DCT through interaction with the transporters TRPM6 and

TRVP5, respectively (**Figure 1**) [16, 85]. Only 5 to 10% of the filtered load of sodium is reabsorbed in the DCT and this is primarily mediated by NCC [23]. Despite this modest contribution to overall sodium reabsorption, the NCC in the DCT together with ENaC in the connecting tubule (CNT) and the collecting duct (CD) fine-tunes the final concentration of sodium chloride in the urine. **This is possible** because it is not affected by the tubuloglomerular feedback [29]. Due to this **property**, NCC plays a pivotal role in extracellular fluid volume and blood pressure control [29].

Structure–function relationship

In humans, NCC is a membrane glycoprotein of 1,021-amino acid residues which resembles the general topology of the sodium-potassium-chloride cotransporters 1 and 2 (NKCC 1 and 2) [70]. NCC is able to form dimers, and it is likely that it functions as a dimer [18, 29]. It consists of 12 putative transmembrane (TM) spanning regions with a central hydrophobic domain (**Figure 2**). Between TM7 and TM8 there is a large extracellular hydrophilic loop that contains two glycosylation sites (N404 and N424) [50]. These glycosylation sites are essential for functioning and surface expression of NCC [40]. When glycosylation is eliminated, thiazide diuretics have much greater access to their binding sites, which suggests that glycosylation blocks the affinity for thiazides [50]. A study that interchanged the transmembrane regions between rat and flounder NCC revealed that affinity-modifying residues for chloride are located within the TM1-7 region [78]. **Especially,** a highly conserved glycine within TM4 plays a crucial role for the affinity of chloride [78]. The TM8-12 region**, distal to the extracellular loop,** is sensitive to thiazides. Site-directed mutagenesis was used to show that a single residue in TM11 defines the different affinity for thiazides between mammalian and flounder NCC [12]. Both **transmembrane regions** appear to have affinity for extracellular sodium. The central domain is flanked by a short amino-terminal domain (N-terminus) and a long carboxy-terminal domain (C-terminus) which are both located intracellularly (**Figure 2**) [29]. The N-terminus of NCC contains several conserved phosphorylation sites including threonine 46, 55 and 60 and serine 73 and 91 in humans [19]. In rats **and mice** these phosphorylation sites correspond to threonine 44, 53, 58 and serine 71, 89**, and 124**. Phosphorylation of NCC appears to determine its activity, especially at threonine 60 in humans or 58 in rat. Indeed, a mutation in threonine 60 is a common cause of Gitelman syndrome [65], the disorder resulting from NCC inactivation (see further). The

development of a knock-in mouse model with this mutation exhibits the Gitelman phenotype and did so because mutant NCC was restricted to the cytosol [138]. Because phosphorylated NCC has thus far only been found in the apical plasma membrane of the DCT, anchoring in the plasma membrane seems necessary for phosphorylation to occur [91]. This suggests that, apart from phosphorylation, the trafficking of NCC from subapical vesicles to the plasma membrane is also important [42].

Physiological functions

NCC is highly regulated by hormones, including aldosterone, angiotensin II, glucocorticoids, estrogen, insulin, norepinephrine, and vasopressin (**Table 1**) [13, 53, 82, 83, 91, 108, 118, 124, 125]. The fact that NCC is regulated by so many different hormones suggests that sodium reabsorption through NCC is an important homeostatic control mechanism. Aldosterone was the first hormone recognized to be capable of activating NCC [53] and the DCT is therefore part of what has been called the "aldosterone-sensitive distal nephron" (ASDN) [77], which was defined to comprise the DCT2, the CNT and the CD [67]. Experimentally, aldosterone upregulates NCC both when it is directly infused or when it is secreted in response to a low sodium diet [14, 53]. The acute effect of aldosterone only involves phosphorylation of NCC [56], whereas the chronic effect also increases the total protein abundance of NCC [53], which likely occurs independent from changes to NCC mRNA levels [1, 69, 119]. The regulation of NCC by aldosterone seems logical, because at least the endportion of the DCT expresses the enzyme 11-beta-hydroxysteroid dehydrogenase II which rapidly inactivates glucocorticoids and hence provides mineralocorticoid-sensitivity to the epithelial cells [6]. The discovery that angiotensin II is also capable of activating NCC was more surprising, because its actions were believed to be confined to the proximal tubule [75]. By adrenalectomizing rats and selectively re-infusing aldosterone or angiotensin II, we were able to dissect the stimulatory effects of angiotensin II and aldosterone on NCC [118]. In a subsequent study we showed that aldosterone did not require angiotensin II for activation of NCC, although the presence of both stimuli led to an additive response [119]. This additive response may be useful to maximize sodium reabsorption during hypovolemia, because this is characterized by elevated plasma levels of both angiotensin II and aldosterone [122]. Increased sodium reabsorption in the DCT by NCC will also decrease the delivery of

sodium to the CNT and CD [42]. Because in these segments sodium reabsorption is electrochemically coupled to potassium secretion, decreased delivery of sodium will help conserve potassium. In line with this, recent studies have shown that angiotensin II directly inhibits ROMK, further contributing to potassium conservation during hypovolemia [49, 129, 140]. The effects of angiotensin II on NCC and ROMK therefore help to understand the aldosterone paradox, the question how aldosterone increases sodium reabsorption during hypovolemia, but potassium secretion during hyperkalemia [3, 122]. Further insight into the aldosterone paradox comes from the effects of potassium on NCC. Recent studies have shown that dietary potassium inhibits NCC [110, 121]. Gastric gavage of potassium in mice increased both urinary potassium and sodium excretion [110]. This response occurred within minutes and was independent from aldosterone, because it could also be induced in aldosterone-deficient animals. In the kidney, this was accompanied by dephosphorylation of NCC (**Figure 4A**). The increased delivery of sodium to the more distal nephron parts facilitates sodium–potassium exchange and therefore kaliuresis. This "potassium-induced natriuresis" appears to constitute an important physiological response. Namely, potassium-induced natriuresis could still be evoked when a high potassium diet was combined with a low sodium diet [121]. This was accompanied by a reduced abundance of total NCC; **phosphorylated NCC was also reduced, although this did not reach statistical significance** (**Figure 4B**). Taken together, these **and other** recent results seem to suggest that when the organism **is** faced with the choice between conserving sodium or secreting potassium, it chooses the latter **[27]**. The focus of future research will be to identify the signal by which dietary potassium induces NCC downregulation. Similar to high dietary potassium, high dietary sodium also suppresses NCC [14]. This response, however, is not as rapid as the one for high potassium diet [110] and involves a decrease in the plasma

membrane abundance of sodium transporters all along the nephron **[28, 137]**. Furthermore, the effect of high dietary sodium on NCC appears to be mediated through aldosterone [14]**, although the effect of high dietary sodium on NCC has not been studies in the absence of aldosterone.** Similarly, chronic metabolic acidosis also increases aldosterone and therefore NCC [26]. Why other hormones such as insulin [13, 57, 108, 109], vasopressin [83, 91, 104], estrogen [125], and norepinephrine [82] regulate NCC is less clearly defined (**Table 1**), but warrants further study given the role of these hormones in normal physiology and human diseases such as diabetes mellitus, obesity, and hypertension.

The NCC signaling cascade

Kinases

The intracellular signaling cascade that controls NCC activity has largely been unraveled in recent years (**Figure 3**). This NCC signaling cascade consists of a multikinase network which includes the kinases WNK, SPAK, OSR1, and SGK1 [130]. More recently, proteins involved in ubiquitylation including Nedd4-2, Kelch-like 3, and Cullin 3 were also found to regulate NCC [2, 7, 68, 99]. Many of these regulatory proteins were identified because mutations in their genes result in familial hyperkalemic hypertension (FHHt, also called pseudohypoaldosteronism type II or Gordon syndrome, see also "Relation to diseases" below). WNKs **appear to** modulate both the "trafficking" and phosphorylation of NCC [41]**, although most of our knowledge has been derived from studies in oocytes.** The regulation of NCC trafficking by WNKs involves a sequential inhibitory cascade, in which KS-WNK1 inhibits WNK1, WNK1 inhibits WNK4 [136], and WNK4 inhibits NCC [134]. The inhibition of NCC by WNK4 is not caused by endocytosis [35], but rather by promoting lysosomal degradation [112]. This inhibition is mediated by the ERK1/2 signaling pathway and the lysosomal targeting receptor sortilin [141,

142]. Interestingly, angiotensin II converts WNK4 from an inhibitor to an activator of NCC [101]. In contrast to WNK4, WNK3 stimulates NCC [97], but its actions are less well-defined. WNK3 and WNK4 not only have divergent effects on NCC, they also antagonize each other. Indeed, it appears to be the ratio between WNK3 and WNK4 that determines the net effect on NCC [135]. The phosphorylation of NCC is mediated by SPAK [79]; several WNKs interact with SPAK and therefore indirectly control the phoshorylation-step of NCC. Interactions between SPAK and WNK1 [79], WNK3 [34], and WNK4 [102] have been reported. However, the brain but not the kidney isoform of WNK3 can activate NCC and does so through a SPAK-independent mechanism [34]. Two isoforms of SPAK have been identified, including full-length SPAK (FL-SPAK) and kidney-specific SPAK (KS-SPAK or SPAK2), of which the latter isoform has low expression levels in the DCT [73]. KS-SPAK, which lacks the kinase domain, inhibits FL-SPAK and OSR1, which are both known to phosphorylate NKCC2. This may explain why in mice the knockout of SPAK results in decreased NCC phosphorylation (absence of full-length SPAK), but increased NKCC2 phosphorylation (no inhibition of FL-SPAK or OSR1 by KS-SPAK) [36, 73].

SPAK deficiency, however, does not completely inhibit NCC phosphorylation [73, 139], suggesting the involvement of other kinases or phosphatases. Both SPAK isoforms are also involved in the stimulatory effect of vasopressin on NCC and NKCC2 [83, 91]. Namely, vasopressin stimulates FL-SPAK in the DCT to phosphorylate NCC, whereas it attenuates KS-SPAK to allow FL-SPAK and OSR1 to phosphorylate NKCC2 [104]. Although SGK1 was first recognized as an activator of ENaC [127], later reports also showed effects on NCC [115]. SGK1 and NCC do not seem to interact directly, but rather through WNK4 and Nedd4-2 [2, 98]. SGK1 phosphorylates WNK4 and this phosphorylation step reduces the inhibition of WNK4 on NCC

[98, 100]. Because SGK1 is sensitive to aldosterone, this pathway appears to be involved in the activation of NCC by aldosterone [100]. The opposite is also true, because SGK1 knockout mice failed to increase NCC activity during a low sodium diet [115].

Ubiquitin ligases

Recent data indicate that Nedd4-2 is yet another player in the pathway by which aldosterone activates NCC (**Figure 3**) [2, 99]. Nedd4-2 was shown to stimulate ubiquitylation of NCC and decreased its activity and surface expression *in vitro* and *in vivo*, while SGK1 prevented these effects [2]. The pathophysiological significance of the regulation of NCC by Nedd4-2 was shown by the generation of inducible nephron-specific Nedd4-2 knockout mice [99]. These mice exhibited salt-dependent hypertension that was characterized by upregulation of total and phosphorylated NCC and sensitivity to thiazides. The deletion of Nedd4-2 also affected ENaC and ROMK, which were down- and upregulated, respectively. This may explain the additional characteristics of these mice, namely that they had a normal Na^+/K^+ balance and were not hyperkalemic. Similar to Nedd4-2, Kelch-like 3 and Cullin 3 are also involved in ubiquitylation, because they are components of an E3 ubiquitin ligase complex. Although mutations in Kelch-like 3 and Cullin-3 cause hyperkalemic hypertension that is reversible with thiazides, these proteins probably do not interact directly with NCC. Instead, Kelch-like 3 binds and ubiquitinates WNK4 and the subsequent degradation of WNK4 would be expected to increase NCC activity (**Figure 3**) [89, 106, 128, 132].

Other signaling molecules

Although the network reviewed above is largely interconnected (**Figure 3**), additional NCC regulatory pathways exist. One example is the regulation of NCC by phorbol esters [54]. This

effect is mediated through Ras guanyl-releasing protein 1 and ERK1/2, which stimulates ubiquitylation and endocytosis of NCC [55]. Furthermore, NCC phosphorylation is not only controlled by kinases but also by phosphatases including phosphatase 4 [33]. Finally, the process that controls the phosphorylation of NCC may also cause less ubiquitylation, thereby increasing the number of cotransporters available for phosphorylation [51].

Relation to human disease

Gitelman syndrome

The clearest demonstration of the functional relevance of NCC comes from human monogenetic diseases that affect NCC function (**Table 2**). Inactivating mutations in *SLC12A3* cause the so-called Gitelman syndrome [107]. Gitelman syndrome is an autosomal recessive disorder that is characterized by hypokalemia, hypomagnesemia, metabolic alkalosis, hypocalciuria, and low to normal arterial blood pressure. Missense mutations account for approximately 59% of the mutations in Gitelman syndrome and compound heterozygosity is common [113, 123]. Gender and the type of mutation contribute to phenotypic variability, with males and patients with homozygous deep intronic mutations exhibiting a **more severe** phenotype [64, 65]. Novel mutations are still being identified in Gitelman syndrome and these genetic defects lead to impaired production, processing, insertion or regulation of the NCC protein (type I, II, III or IV mutations) [32, 113, 123]. Although Gitelman syndrome is usually a relatively benign disorder that often remains subclinical for many years, a recent report suggests that the electrolyte disorders associated with Gitelman syndrome may result in chronic kidney disease and glucose intolerance [113]. On the other hand, heterozygous mutations in NCC may prevent hypertension and cardiovascular diseases [52] and improve bone-density [17] likely because they induce mild sodium excretion and a positive calcium balance. NCC knockout mice recapitulate the phenotype of Gitelman syndrome, although some features become manifest only when the animals are challenged [73, 80, 105, 139]. In line with the current model of NCC regulation (**Figure 3**), genetically modified mice with overexpression of WNK4, deficiency in SPAK or its kinase domain, also exhibit features of Gitelman syndrome [61, 88, 94, 139], although such mutations have not been identified in humans. Gitelman syndrome should be differentiated from the more

severe Bartter syndrome, which results from mutations affecting NKCC2, and is characterized by earlier onset of symptoms and hypercalciuria instead of hypocalciuria [47]. An intermediary phenotype is caused by mutations in *CLCNKB* which encodes a basolateral chloride channel that is expressed in both the thick ascending limb and the DCT (**Table 2**) [58]. **Disturbed basolateral chloride efflux will indirectly impair apical NaCl transport through NKCC2 and NCC.**

Familial hyperkalemic hypertension

Familial hyperkalemic hypertension (FHHt) is the "mirror image" of Gitelman syndrome because, in addition to hyperkalemia and hypertension, it is characterized by hypercalciuria and metabolic acidosis [37]. Surprisingly, no activating mutations in *SLC12A3* have been reported. Overexpression of NCC in transgenic mice also failed to induce hyperkalemic hypertension, but this may have been due to the fact that phosphorylated NCC was not increased [74]. Similarly, increasing NCC activity by inactivating KS-WNK1 was also not sufficient to cause hyperkalemic hypertension, because it was associated with a compensatory decrease in ENaC [38]. Instead, familial hyperkalemic hypertension is caused by mutations in the genes encoding WNK1, WNK4, Kelch-like 3 or Cullin-3, which result in overactivity of NCC (**Table 2**) [7, 68, 131]. Because the WNKs regulate other transporters than NCC alone, these effects may also contribute to the FHHt-phenotype [37]. Intronic deletions in the WNK1 gene cause overexpression of WNK1 and therefore more inhibition of WNK4. The inhibition of WNK4 will relieve the inhibition of SPAK and will activate NCC (**Figure 3**). Similarly, missense mutations in the WNK4 gene give rise to a mutant protein that no longer inhibits SPAK resulting in NCC activation. Mutations in *KLHL3* can be dominant or recessive and homozygous or heterozygous,

while the identified mutations in *CUL3* were dominant, heterozygous and often *de novo* [7]. Mutations in *KLHL3* and *CUL3* seem to abrogate ubiquitylation of targets normally bound by KLHL3 including WNK4 [106]. Of the different mutations causing FHHt, *CUL3* mutations have the most severe phenotype, including the youngest onset of hypertension and the highest serum potassium [7]. Another illustration that NCC activation is the final common pathway of the FHHt mutations is the fact that these disorders are all exquisitely sensitive to treatment with thiazide-type diuretics [71].

Drugs influencing NCC activity

Drugs inhibiting NCC

Several commonly used drugs inhibit or stimulate NCC activity (**Table 1**). The sensitivity of NCC for thiazide diuretics is well known and characterizes this cotransporter. Thiazides, however, do not exclusively inhibit NCC, but also the sodium-dependent bicarbonate-chloride cotransporter in the cortical collecting duct [63]. Thiazide diuretics were incidentally discovered while searching for better carbonic anhydrase inhibitors [86]. They were implemented clinically in 1958, long before it became apparent that their primary target is NCC [81]. Thiazide diuretics are the logical drug of choice for diseases with NCC overactivity such as the rare disorder FHHt. However, the clinical indication for thiazide diuretics is much broader. In fact, thiazide diuretics are still among the most commonly used drugs to treat hypertension worldwide. Although the natriuretic effect of these drugs undoubtedly contributes to their antihypertensive effect, this response also seems to be translated to an effect on vascular tone [9-11]. In addition, thiazides may directly cause vasodilation possibly through vascular potassium channel activation [92]. In addition to hypertension, thiazide diuretics can also be used for sodium-retaining disorders such as heart failure, liver cirrhosis, nephrotic syndrome, and chronic kidney disease [8]. Because thiazide diuretics act in the tubular lumen, their action requires successful delivery to the DCT. This is mediated by active secretion of thiazide diuretics in the proximal tubule through organic anion transporter and multidrug resistance-associated protein 4 [39, 114]. This explains why a reduction in glomerular filtration rate results in reduced efficacy of thiazide diuretics. Patients with Gitelman syndrome also demonstrate a dramatically impaired natriuretic response to thiazides, a feature that may be used diagnostically [15]. The side-effect profile of thiazide diuretics resembles Gitelman syndrome with the exception of hyponatremia, which is only seen with thiazides [22, 24, 48].

Drugs stimulating NCC

Drugs *stimulating* NCC activity have also been identified recently and they include the calcineurin inhibitors cyclosporine and tacrolimus (**Table 1**) [44, 76]. Calcineurin inhibitors are potent immunosuppressive drugs that are used clinically to prevent rejection after transplantation and sometimes in autoimmune disorders. Although cyclosporine and tacrolimus have different intracellular binding proteins, they both activate NCC, and this effect therefore appears to be a class effect [45]. Again, cyclosporine and tacrolimus do not seem to activate NCC directly, but influence the WNKs [76]. This possibility was already suggested by the side-effect profile of these drugs, which is similar to the phenotype of FHHt. Of interest, calcineurin is a protein phosphatase and protein phosphatases were recently shown to regulate NCC [33]. We were able to illustrate the clinical relevance of NCC activation by tacrolimus by linking it to hypertension [44]. That is, tacrolimus failed to induce hypertension in NCC knockout mice, whereas it caused more severe hypertension in NCC transgenic mice. Furthermore, a thiazide diuretic caused a larger urinary chloride excretion in patients on tacrolimus than in healthy volunteers or patients on sirolimus (a different immunosuppressant). This enhanced chloriuretic response to a thiazide was interpreted as an indication of increased transporter activity [15]. Also, the expression of total and phosphorylated NCC was increased in the kidney biopsies of patients treated with tacrolimus. Of interest, a model of cyclosporine nephrotoxicity showed the opposite effect on NCC, but this was attributed to inactivation of the renin-angiotensin system [62]. Furosemide, which blocks sodium transport in the thick ascending limb but not in the DCT, also increases NCC abundance. This effect is likely caused by the loop-diuretic induced enhanced sodium delivery to the DCT and the activation of the renin-angiotensin system [1]. The up-regulation of NCC likely compensates for the furosemide effect and hence may contribute to the phenomenon

of loop-diuretic resistance which frequently develops during chronic treatment. As such it provides the rationale for the combination of a loop-diuretic with a thiazide to overcome diuretic resistance [8]. Furthermore, recent studies in rats suggested that the cytostatic drug cisplatinum decreases renal NCC abundance. However, this effect is likely attributed to a general toxic effect on DCT cells, because also the expression of other DCT-specific proteins such as the magnesium channel TRPM6 and the calcium- and magnesium-binding protein parvalbumin were decreased [116].

Perspectives

In this review we have highlighted several exciting new roles of NCC in sodium, potassium, and blood pressure regulation. These new insights have partly solved longstanding questions in physiology, but at the same time raise new questions. For example, although the model of NCC regulation is gaining more clarity (**Figure 3**), some of the interactions are still not well understood or even controversial. For example, WNK4 seems to be a negative regulator of NCC under some conditions, but may become a positive regulator in others [72]. The inhibition of NCC by dietary potassium may be mediated by WNK4 [87, 110, 120], but leaves the question open through which signal DCT cells "sense" dietary potassium. Pathophysiologically, the role of NCC in "essential" hypertension will likely remain a focus of future studies. The recent linkage of Cullin-3 and Kelch-like 3 to FHHt begs the question whether polymorphisms in these genes exist and whether they may contribute to human hypertension. The discovery that calcineurin inhibitors stimulate NCC to cause hypertension warrants a clinical study to evaluate whether thiazide diuretics are effective drugs to treat this side effect [44-46]. Especially for translational studies it will be important to have a measure of NCC activity *in vivo.* In addition to testing the response to a thiazide diuretic [15], the analysis of NCC in so-called urinary exosomes holds promise [43]. Urinary exosomes are vesicles derived from renal tubular epithelial cells that are thought to reflect the metabolic profile of these cells [93]. We recently showed that the abundance of phosphorylated NCC in urinary exosomes correlated with elevated aldosterone levels in animals and humans [117]. Although this review focused on NCC, sodium excretion by the kidney depends on many other sodium transporters, including NHE3, NKCC2, ENaC, and pendrin. Stimuli that activate NCC, sometimes also activate these other transporters, but an opposite, compensatory response may also occur [38, 84, 99]. In conclusion, the role of NCC in normal physiology and in the pathophysiology of hypertension is expanding and will

likely continue to do so in coming years. To fully grasp the potential of these insights for the treatment of human disease will likely require a more complete understanding of the molecular physiology of this fascinating cotransporter.

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Table 1: Hormones, metabolic stimuli and drugs influencing NCC activity

	Stimulus	Effect	References
Hormones	Angiotensin II	Stimulatory	[118]
	Aldosterone	**Stimulatory¶**	[53]
	Glucocorticoids	Stimulatory	[124]
	Vasopressin	Stimulatory	[83, 91, 104]
	Insulin	Stimulatory	[13, 57, 108, 109]
	Estrogen	Stimulatory	[125]
	Norepinephrine	Stimulatory	[82]
Metabolic stimuli	Dietary potassium	Inhibitory	[110, 120]
	Dietary sodium*	Inhibitory	[14, 103]
	Dietary magnesium*	Stimulatory	[25]
	Acidosis*	Stimulatory	[26]
Drugs	Thiazide diuretics	Inhibitory	[16, 23]
	Furosemide*	Stimulatory	[1]
	Tacrolimus	Stimulatory	[44]
	Cyclosporine†	Stimulatory	[76]
	Cisplatin	Inhibitory	[116]

Footnote: ¶ When high dietary K increases aldosterone, NCC may be inhibited; * Some of these effects may be mediated through aldosterone; † In a model of cyclosporine nephrotoxicity,

NCC was downregulated, but this may be attributed to kidney failure and reduced renin-angiotensin activity [76].

Table 2: Characteristics of mutations causing Gitelman syndrome or familial hyperkalemic hypertension

Disease	**Gene**	**OMIM gene**	**Human chromosome location**	**Encoding protein**	**Major phenotype**	**Selected references**
Gitelman (OMIM 263800)	*SLC12A3*	600968	16q13	NCC	Hypokalemic hypotension	[107]
	CLCNKB	602023	1p36.13	CLCNKB	Variable	[58]
FHHt* (OMIM 179820)	*WNK1*	605232	12p13	WNK1	Hyperkalemic hypertension	[131]
	WNK4	601844	17q21	WNK4	Hyperkalemic hypertension	[131]
	KLHL3	605775	5q31	Kelch-like 3	Hyperkalemic hypertension	[7]
	CUL-3	603136	2q36	Cullin-3	Hyperkalemic hypertension	[7, 68]

Abbreviation: * FHHt, familial hyperkalemic hypertension; OMIM, online Mendelian inheritance in man.

Figure legends

Figure 1: Model of transcellular transport in the early distal convoluted tubule (DCT). A kidney tubule is shown schematically on the left indicating the locations of DCT type 1 and type 2 (DCT1, DCT2) and the connecting tubule (CNT). The sodium chloride cotransporter (NCC) is primarily expressed in DCT1. A model of transcellular transport in DCT1 is shown on the right, including the apical transporters NCC and transient receptor potential channels TRPV5 (a calcium channel) and TRPM6 (a magnesium channel). On the basolateral side the sodium potassium ATPase pump is shown as well as the chloride channel ClC-Kb and the sodium-calcium channel NCX1. This figure was adapted from [5, 59].

Figure 2: Putative structure of the sodium chloride cotransporter. The twelve transmembrane domains are shown including the hydrophilic loop with the two glycosylation sites. A detailed image of the N-terminus is provided on the left showing the binding sites of γ-adducin and the kinases SPAK/OSR1. This figure was reproduced and adapted from [19] with kind permission.

Figure 3: Current model of sodium chloride cotransporter regulation by kinases and ubiquitinases. The various interactions of the NCC regulatory pathway are shown as arrows (stimulatory) or as lines ending with perpendicular lines (inhibitory). Phosphorylation is indicated with the symbol “P”, whereas ubiquitylation is shown as “U”. SPAK/OSR1, WNK4, kidney-specific WNK1 (KS-WNK1) and long WNK1 (L-WNK1, also called WNK1) are kinases. The role of mutant WNK4 in familial hyperkalemic hypertension (FHHt) is also shown, which overrides the inhibitory effect of wild-type WNK4 on SPAK/OSR1. Nedd4-2 is a ubiquitinase, while Cullin-3 and Kelch-like 3 interact in a ubiquitylation complex that likely

ubiquitinates WNK4. **Although WNK3 has been shown to interact with WNK4 and SPAK [90, 135], its precise role in NCC regulation remains less clear and we therefore decided not to include it.** See text for further details.

Figure 4: Inhibition of the sodium chloride cotransporter by dietary potassium **("high K")**. The results of our recent studies on the inhibitory effect of dietary potassium on NCC are shown [110, 121]. Panel A shows that dietary potassium acutely downregulates phosphorylated NCC but not total NCC. Conversely, panel B shows that a chronic high potassium diet primarily decreased total NCC, but phosphorylated NCC less so. **The high potassium diet was 2% and 5%, respectively, and the low sodium diet was < 0.001% [110, 121].**

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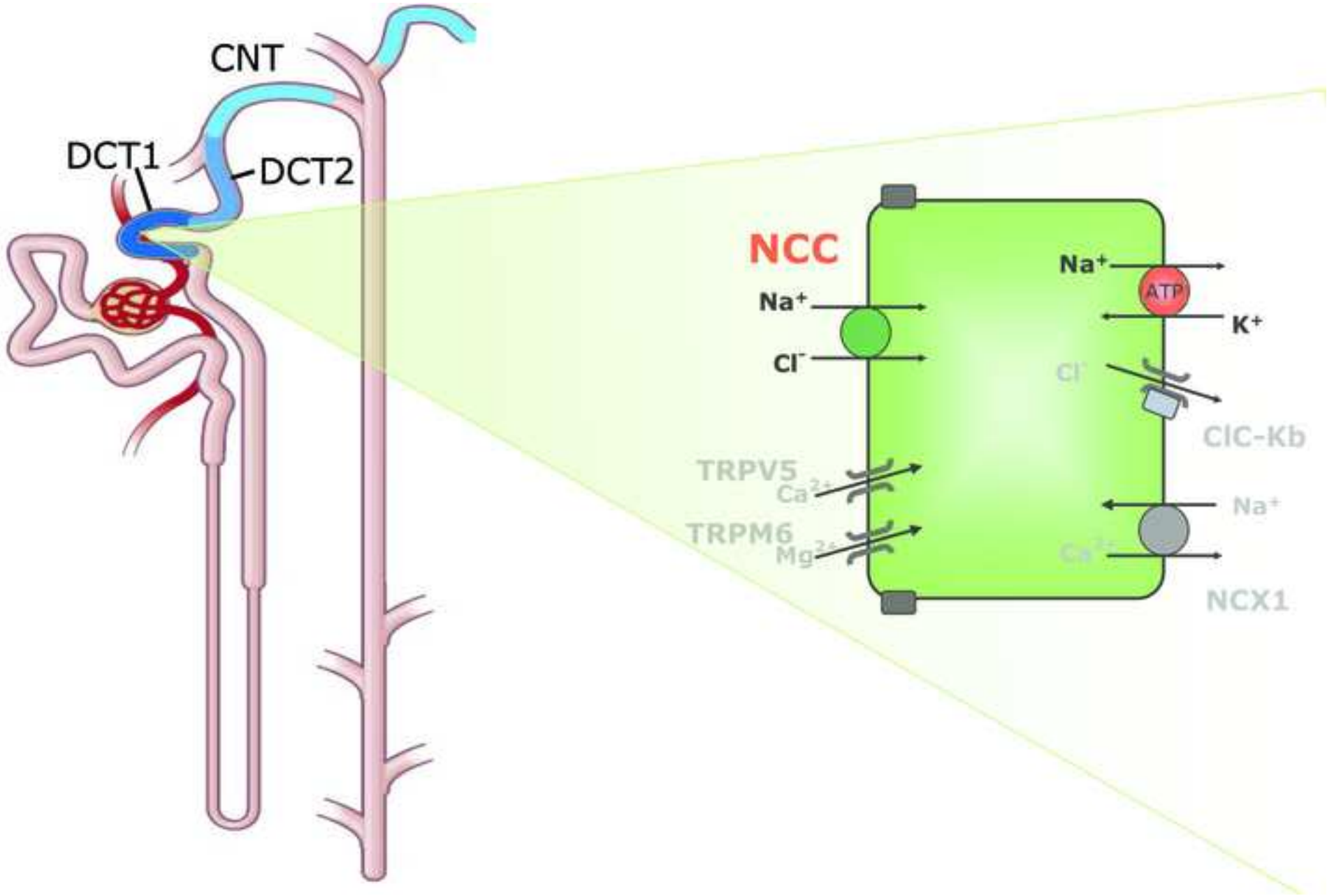

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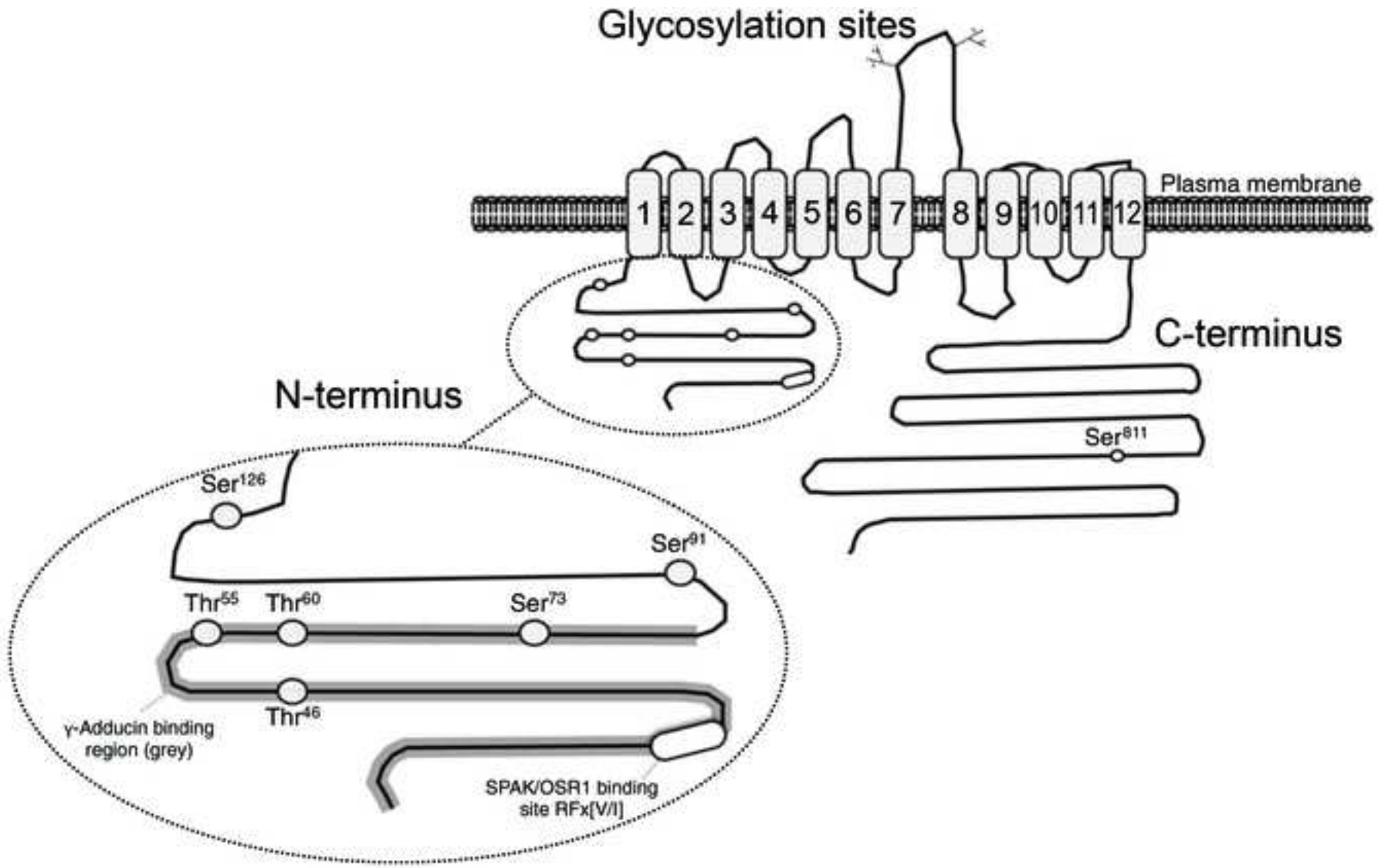

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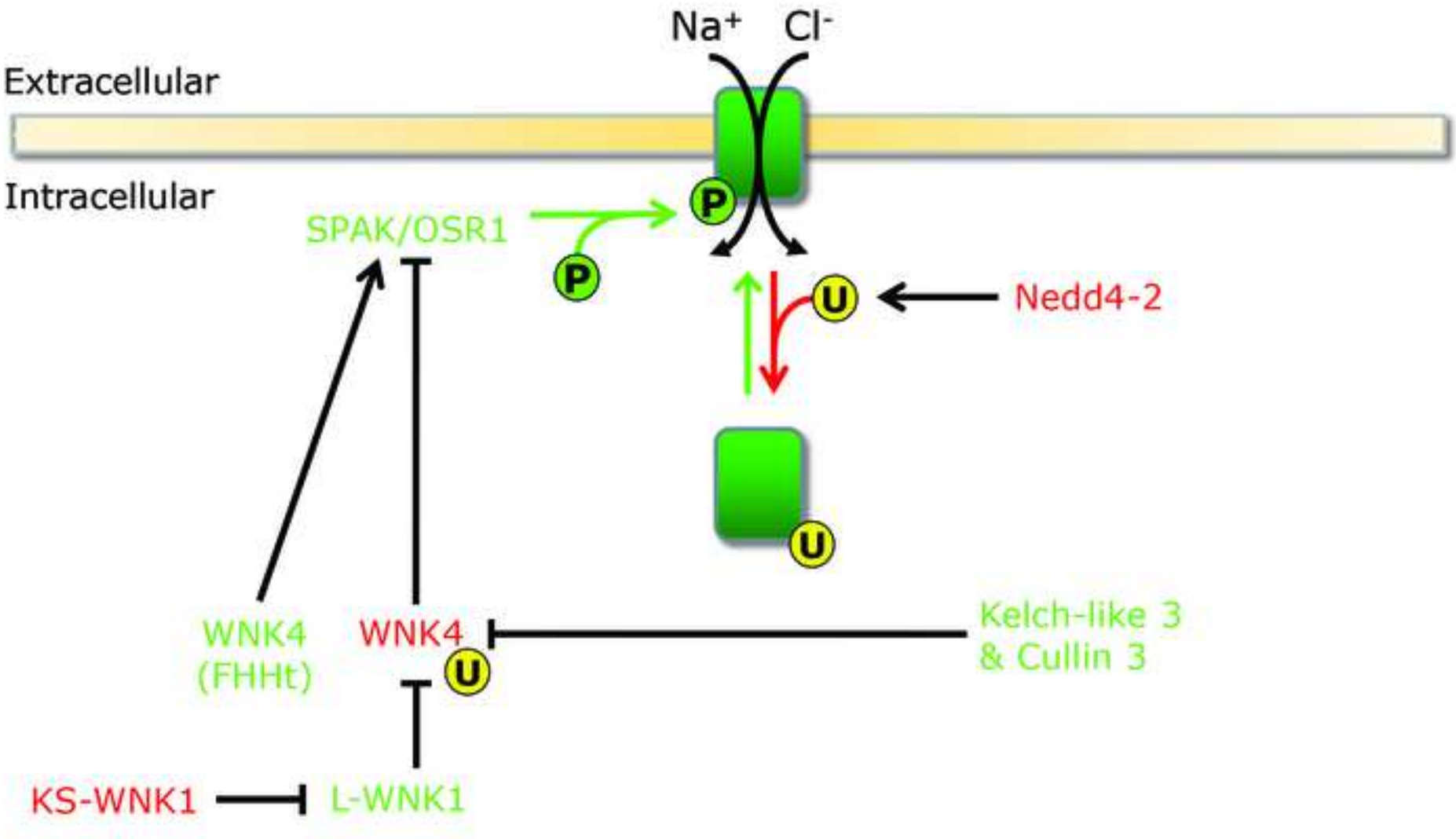

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